Expression Profiles of opomir miR-21 and Tumor Suppressor let-7a in the Progression of Opisthorchiasis-Associated Cholangiocarcinoma

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Abstract

Altered miRNA expression could be a determinant of cancer development and/or progression. We aimed to study the role of oncomir miR-21 and tumor suppressor let-7a in the genesis of Opisthorchiasis-associated cholangiocarcinoma (CCA). The results showed that miR-21 was up-regulated while let-7a was down-regulated during cholangiocarcinogenesis in the hamster model and also in human CCA samples. The expression level of miR-21 had an inverse correlation with the mRNA level of its target RECK, a metastasis suppressor, in human CCA. Knockdown of miR-21 of KKU100 CCA cells significantly increased the mRNA level of RECK and suppressed the wound-induced migration of CCA cells. Our data suggest that miR-21 is one key molecule playing crucial roles in the CCA growth and metastasis. Manipulation of miRNA expression offers a potential avenue of CCA therapy.

Keywords: miR-21 - let-7a - RECK - Opisthorchis viverrini - cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is an aggressive type of cancer that originates from the bile duct epithelium. Cases of CCA are increasing worldwide (McLean and Patel, 2006). In Thailand, the highest incidence is in the northeast region and is primarily associated with chronic infection with the liver fluke, Opisthorchis viverrini (Ov) (Haswell-Elkins et al., 1994; Sripa et al., 2007). At present, only surgical resection of all detectable tumors leads to an improvement of the five-year survival. Surgical resection is often incomplete and typically results in subsequent local recurrence and metastasis. In order to prevent post-surgical recurrence, chemotherapy is a preferred adjunctive treatment. Accordingly, molecular mechanisms of CCA genesis and progression need to be studied to identify the targets for chemoprevention and treatment.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate the expression of target genes by interacting with the 3’UTR and promoting translational repression or degradation of mRNAs (Peek and Blaser, 2002; Pillai, 2005). In recent years, abnormalities in miRNA expression have been identified in the progression of various cancers and consequently have been proposed as potential targets for anticancer therapies (Croce, 2008). miRNAs have been shown to function as both tumor suppressors or oncogenes in various cancers (Kent and Mendell, 2006; He et al., 2007). Among these oncogenic miRNAs, miR-21 has been identified as the top-rank upregulated miRNA in various types of cancers as reviewed by Krichevsky and Gabriely (2009). miR-21 has been linked with inflammation-related cancers by activating its expression via inflammatory mediators such as interleukin 6 (IL6) (Schetter et al., 2010) through a signal transducers and activators of transcription 3 (STAT3)-dependent mechanism (Loffler et al., 2007) and stimulating maturation via transforming growth factor β (TGFβ) through the Smads signaling cascade (Hata and Davis, 2009). miR-21 emerges as a principal regulator targeting to miRNAs of several tumor suppressor genes.

One of well-characterized miR-21 targets that has recently been reported is the metastasis suppressor RECK (Reversion-inducing cysteine rich protein with Kazal motifs) (Gabriely et al., 2008; Reis et al., 2012; Zhang et al., 2008). RECK functions as the inhibitor of metalloproteinases (MMPs) that play roles in regulation of extracellular matrix and basement membrane degradation, which is the key step of cancer metastasis (Oh et al., 2001). Recently, we demonstrated that RECK was downregulated in CCA tissues and cell cultures and the RECK level was inversely correlated with MMP-2 and MMP-9 expressions. Low level of RECK protein was associated with poor survival of CCA patients (Namwat et al., 2011). Knockdown of RECK using siRNA resulted

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in the elevation of the gelatinase activity of MMP-2/-9 and increasing CCA cell invasion (Namwat et al., 2011).

Down-regulation of tumor suppressor miRNAs is frequently found in cancer progression. let-7a, the first identified miRNA originally isolated from Caenorhabditis elegans, was found to act as a tumor suppressor directly regulating RAS and HMGA2 oncogenes (Johnson et al., 2005; Lee and Dutta, 2007; Qian et al., 2009). Transfection of let-7a into the MCF-7 breast cancer cell line suppressed migration and invasion through down-regulation of C-C chemokine receptor type 7 (Kim et al., 2012). let-7a, however, has not been elucidated its profile in the carcinogenesis of CCA that is associated with the liver fluke infection.

In the present study, we examined the expression patterns of miR-21 and let-7a in the genesis of Ov-associated CCA in both animal model and human surgical materials. Effects of miR-21 knockdown on RECK expression and CCA cell migration were also determined to exhibit an encouraging therapeutic approach for CCA.

Materials and Methods

Animals and tumor induction

Frozen liver samples were obtained from the previous study of the induction of CCA in male Syrian golden hamsters by Ov metacercariae infection combined with N-nitrosodimethylamine (NDMA) treatment (Detchakhamphu et al., 2010). The animal experiments were conducted according to the guidelines of the National Committee of Animal Ethics. The protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Thailand.

Patients and samples

Ten pairs of tumor and non-tumorous tissues of frozen intrahepatic CCA specimens from primary tumors of patients, collected from 2002 to 2004, were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Written informed consent was attained from all patients in accordance with the Declaration of Helsinki and its later revision. The Human Research Ethics Committee, Khon Kaen University (#HE4320 and #HE471214), approved the research protocol.

Cell culture, chemicals, and reagents

The KKU100 CCA cell lines used in this study was established at the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. The cell line was cultured in HAM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C with 5% CO₂. All other chemicals used were of analytical grade.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from tissues with Trizol reagent (Invitrogen, Carlsbad, CA). To analyze the expression levels of miR-21 and let-7a, miRNA specific reverse transcription was performed with 100 ng total RNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer for miR-21, let-7a. U6 small nuclear RNA was used for normalization. qPCR was performed using the TaqMan® microRNA Assay Kit in an ABI7500 Real Time PCR System (Applied Biosystems).

To determine the expression level of RECK mRNA, 2 µg of RNA was reverse transcribed into cDNA using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, CA) and random hexamers (Promega, Madison, WI). qPCR was performed with TaqMan® Gene Expression Assay reagent kits (Applied Biosystems) in an ABI7500 Real Time PCR System (Applied Biosystems). Expression levels of target genes were calculated relative to that of GAPDH.

Transient knock down of miR-21

The KKU100 CCA cells (3×10⁴) with high endogenous miR-21 gene expression were seeded in a 12-well plate for 24 h prior to transfection with the anti-miR™ miRNA inhibitor specific for miR-21 and validated non-targeting siRNAs (negative controls) (Ambion, Foster City, CA) using RNAiMAX Transfection Agent (Invitrogen), according to the manufacturer’s instructions. The final concentration of anti-miR-21 was 41 nM. Cells were cultured for 48 h to achieve complete transfection. Levels of miR-21 and RECK mRNA were determined by qRT-PCR assay.

Wound-induced cell migration assay

To investigate wound-induced CCA cell migration, a wound healing assay was performed as previously described (Lampugnani, 1999) with some modifications. Cells were seeded and allowed to grow until 100% confluence was reached. Anti-miR-21 was introduced 48 hours to knockdown the endogenous miR-21 prior to the start of the assay. A wound was then generated by scratching a straight line on the culture. Cells were washed to remove dislodged cells and anti-miR-21 containing medium was replaced with normal medium. Migration of cells into denuded areas was monitored and visualized by a phase contrast microscope with a digital camera (Axiovert 40, Germany).

Statistical analysis

Results were presented as the mean±SD and evaluated for the statistical significance using the independent t-test or Mann-Whitney test if data did not assume Gaussian distributions. The significant difference was considered at P≤0.05.

Results

Expression pattern of miR-21 and let-7a in the carcinogenesis of Ov plus NDMA-induced hamster CCA model

Our results demonstrated that the expression of miR-21 was significantly increased in hamster liver tissues after Ov plus NDMA administration on week 8, 12 and 16 and persistently upregulated in hamsters up to week 24 when
the CCA developed (Figure 1A). let-7a expression in Ov plus NDMA induced CCA tissues was found to be the highest level on week 3 compared with the untreated group (Figure 1B). Expression levels of let-7a were persistently increased on week 8, 12 and 16 albeit at lower levels than those on week 3 (Figure 1B). It was noted that the level of let-7 was not markedly upregulated on week 24 and the level was lowest compared with other treated groups. In overall, the expression of miR-21 was increased while those of let-7 were decreased along the carcinogenesis.

Expression levels of miR-21 and let-7a in clinical samples

The expression profiles of miR-21 and let-7a in the human CCA tissues (n=10 cases) were analyzed using qRT-PCR. In Figure 2, the results showed that miR-21 expression level in tumor tissues was significantly higher than those in non-tumorous tissues. In contrast, the expression of let-7a was markedly low in both tumor and non-tumorous tissues.

Downregulation of the target of miR-21 in human CCA tissues

To elucidate the significance of overexpression of miR-21, the RECK mRNA level was measured in clinical samples. Anti-miR-21 was transfected into the KKU100 CCA cell line to knockdown the endogenous miR-21. The expression levels of miR-21, RECK mRNA, and wound-induced migration were measured. The results showed that knockdown of miR-21 significantly increased the expression of RECK mRNA and decreased wound-induced migration in the human CCA cell line.
the miR-21, expression levels of RECK mRNA, a target of miR-21, was examined in human CCA tissues (n=10 cases) using qRT-PCR. As shown in Figure 3, RECK mRNA was less expressed in tumor tissues than non-tumorous tissues albeit non-significant difference.

**Effects of miR-21 knockdown on RECK expression and the migration of the KKU100 cell line**

KKU100 cells transfected with anti-miR-21 showed 50% suppression of miR-21 mature sequence (Figure 4A). Upon 48 h of transfection, the miR-21-depleted KKU100 cells showed significantly elevated levels of RECK mRNA (P<0.05) (Figure 4B) and retarded wound-induced migration at 32 h (Figure 4C).

**Discussion**

Altered expression of microRNAs has been widely described as a key modification in many different cellular processes including metabolism, apoptosis, differentiation, and development (Wang and Lee, 2009). In cancer, microRNA sequences have been discovered and function as oncogenic factors and tumor suppressors that play roles in networking of tumor progression and metastasis processes. Among those, miR-21 has been shown to be upregulated in many cancers such as glioma (Chan et al., 2005), breast cancer (Zhu et al., 2008), ovarian cancer (Iorio et al., 2007), hepatocellular carcinoma (Meng et al., 2007), colorectal cancer (Asangani et al., 2008), prostate cancer (Volina et al., 2006) and CCA (Chen et al., 2009; Selaru et al., 2009). Let-7 was first identified in *Caenorhabditis elegans* (Reinhart et al., 2000). It is less expressed in the embryonic stage of development, but becomes more abundant in later stages of development (Lee et al., 2005). Previous reports demonstrated that a reduced expression level of let-7 was commonly found in lung tumors (Takamizawa et al., 2004), colon cancer (Akao et al., 2006) and prostate cancer (Dong et al., 2010). Let-7 retards cellular proliferation by down-regulating the oncogenes RAS/c-MYC and HMGA-2 at the translational level (Johnson et al., 2005; Lee and Dutta, 2007). Our results revealed that the genesis of Ov-associated CCA increased the ratio of oncomir per tumor suppressor miRNAs presenting as upregulation of miR-21 but low-expression of let-7a. CCA is a chronic inflammation-related cancer that presents the high level of IL-6 (Sripa et al., 2012), possibly leading to the stimulation of miR-21 expression (Schetter et al., 2010). Our study also demonstrated the Ov-associated CCA suppressed the expression of let-7a in which RAS activation (Petmitr et al., 1998) is, possibly due to, in part, down-regulation of let-7a.

miR-21 defined as an oncomir regulates the protein translation of mRNA targets such as the metastasis suppressor RECK (Gabriely et al., 2008; Reis et al., 2012; Zhang et al., 2008), proapoptotic protein PDCD4 (Frankel et al., 2008) and tumor suppressor PTEN (Meng et al., 2007). The roles of miR-21 studied in cancers are to regulate cellular proliferation, migration and invasion (Krichevsky and Gabriely, 2009). In the present study, we demonstrated the role of miR-21 on the migration ability of CCA cell culture. Knockdown of miR-21 resulted in reduced wound-induced migration ability of KKU100 cells and induction of RECK expression level, as similar to the results observed for glioma, prostate cancer and gastric cancer cells. Therefore, suppression of miR-21 in CCA cells may be a potential therapeutic approach for CCA therapy. Further investigation on the insight of molecular mechanisms by which miR-21 regulates the networking of CCA progression is underway.

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**References**


fluke-induced cholangiocarcinogenesis in hamsters. Mutat Res, 691, 9-16.


